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Award Number: DAMD17-01-1-0191

TITLE: Analysis of Pro-Apoptotic and Antiangiogenic Activity of CC3 in Breast Cancer Cells

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REPORT DATE: August 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2003	Final (16 Jul 2001 - 15 Jul 2003)

4. TITLE AND SUBTITLE
Analysis of Pro-Apoptotic and Antiangiogenic Activity of CC3 in Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-01-1-0191

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

CC3 was identified as a metastasis suppressor protein in vivo. This laboratory demonstrated that CC3 impairs apoptotic resistance of cells derived from aggressive tumors and inhibits production of angiogenic factors by these cells. CC3 expression was introduced into two breast carcinoma cell lines derived from metastatic tumors and expressing very low levels of this protein. Expression of exogenous CC3 lead to enhancement of their apoptotic responses to growth factors withdrawal and treatment with cytotoxic drugs. However, there was no effect of CC3 expression on angiogenic activity of breast cancer cells which was very low even prior to introduction of CC3.
To understand the mechanisms of the pro-apoptotic activity of CC3, we have conducted analysis of cellular proteins that interact with CC3. Mass-spectormetric analysis identified five importins beta and one exportin, i.e. proteins that serve as nuclear transport receptors. We have analyzed the possible role of CC3 in regulation of nuclear import of proteins. Importantly, by using specific mutants of CC3, we have shown that the inhibitory activity of CC3 in nuclear import is tightly linked to its ability to induce apoptosis. We suggest that CC3 inhibits nuclear import under conditions of stress leading to apoptosis and present data that in breast cancer cells high levels of CC3 could impair survival in response to stress.

14. SUBJECT TERMS
Angiogenesis, apoptosis, nuclear import

15. NUMBER OF PAGES
36

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Development of aggressive and metastatic phenotypes in tumors largely depends on acquisition by tumor cells of ability to support angiogenesis and resist apoptosis. Human gene CC3 is able to negatively affect both of these features of metastatic cells. CC3 is a metastasis suppressor of variant small cell lung carcinoma (vSCLC) and a mouse melanoma *in vivo*(7, 12). We have identified two likely mechanisms whereby CC3 acts as a metastasis suppressor. First, forced expression of CC3 restores apoptotic responses of tumor cells to a wide variety of signals(12, 15). Since the ability to resist apoptosis is an essential part of metastatic phenotype, the pro-apoptotic function of CC3 is likely to contribute to suppression of metastasis. Second, we have found that expression of CC3 in tumor cells results in suppression of their angiogenic activity *in vitro* (11). Enforced expression of CC3 in tumor cells leads to reduced expression of angiogenic stimulatory factors and increase in antiangiogenic factor production. Since angiogenesis is necessary for the development of metastasis, ability of CC3 to suppress angiogenesis is crucial in its metastasis suppressing ability. We proposed to examine how enforced expression of CC3 in breast carcinoma cell lines with low levels of CC3 might affect their ability to undergo apoptosis and inhibit angiogenesis. Our results show that the ability of CC3 to inhibit nuclear import is key to its pro-apoptotic activity.

BODY

Task 1. To examine effects of CC3 on angiogenic properties of breast cancer cells.

We have examined angiogenic properties *in vitro* of three breast carcinoma cell lines: two lines with almost undetectable levels of CC3 (MDA-MB-361 and 468) and one with moderate levels of CC3 protein (DU4475). The assays were conducted with conditioned medium collected from these cell lines and cultures of primary human umbilical vein endothelial cells (HUVEC). Proliferation of HUVEC cultures was examined in a tritiated thymidine incorporation assay; conditioned medium from small cell lung carcinoma and glioblastoma cell lines was used a positive control for angiogenic properties as described (11). However, we have found that the angiogenic activity *in vitro* of breast carcinoma cell lines is not significant (data not shown). Conditioned media from MDA-MB361, 468 and DU4475 did not induce proliferation of HUVEC cells over background values. Therefore we could not proceed with analysis of a potential inhibitory effect of CC3 on angiogenic properties of breast carcinomas *in vitro*.

Task 2. To identify angiogenic modulators whose expression levels are affected by CC3 (Months 15 - 24).

This task could not be accomplished because, as explained above, breast carcinoma cell lines examined under Task 1have no appreciable angiogenic activity *in vitro*.

Task 3. Examine the effect of exogenous CC3 expression on apoptosis of the breast cancer cells (Months 9 – 24).

Expression of CC3 in breast carcinoma cell lines increases their sensitivity to apoptotic signals.

Expression vector for CC3 was introduced into two breast carcinoma cell lines, MDA-MB-468 and 361, both derived from metastatic lesions and containing very low levels of endogenous CC3. About 40 clones were isolated from each line and only two clones in each line were found to express moderate levels of exogenous CC3. These clones were subjected to treatment with several inducers of

apoptosis; in all cases the level of death was found to be higher in CC3-expressing clones compared to neo controls (Table 1).

Table 1. Expression of CC3 predisposes breast cancer cells to apoptosis in response to a variety of treatments

	MDA-MB-468 NEO	MDA-MB-468 CC3	MDA-MB-361 NEO	MDA-MB-361 CC3
Serum withdrawal*	9.0	19.2	1.1	3.9
Etoposide 50 mg/ml	34.5	62.1	5.8	18.6
Taxol 50 nM	36.1	55.1	5.5	20.1
Worthmannin 10 nM	8.1	19.1		

* All treatments were for 24 hours. Data are shown as % of cells with subdiploid DNA content and are average of at least two experiments.

These data show that CC3 acts as a pro-apoptotic protein in breast carcinoma cell lines in a manner that is not signal-specific.

We have therefore conducted experiments aimed at identification of the mechanisms through which CC3 exerts its pro-apoptotic action. Our findings are briefly described below.

CC3 inhibits nuclear import *in vitro* and *in vivo*. In an effort to elucidate the molecular mechanisms underlying the pro-apoptotic activity of CC3, we have identified cellular proteins that interact with CC3. An identical set of proteins from HeLa and MCF7 cells specifically interacted with CC3. Surprisingly, out of eight cellular proteins identified by mass-spectrometry in association with CC3, six belonged to the family of importins b of nuclear transport receptors.

The bi-directional transport of macromolecules between the nucleus and cytoplasm through the nuclear pore complex (NPC) is an active and regulated process. It depends on carrier proteins known as importins and exportins (or karyopherins) that compose the importin β family with 22 members in humans (reviewed in (14)). Members of this family can interact with their cargoes directly or use adapter proteins. Importin β1, for example, mediates nuclear import of proteins containing a basic nuclear localization signal (NLS) by using importin a (1). Importin β2 recognizes the M9-type nuclear localization sequences, and imports M9-containing RNA binding proteins without requiring an adapter molecule (9).

Importins β mediate interactions of transport complexes with the NPC. Importins β also bind to the small GTPase Ran that controls the directionality of the transport (reviewed in (4)). Importins β bind specifically to the GTP loaded form of Ran found in the nucleus. The importin-cargo complex docks at the cytoplasmic face of the NPC, followed by the translocation across the NPC via interactions with nucleoporins (2). At the terminal nucleoporin, the cargo is released from importin into the nucleus by RanGTP, and RanGTP bound importin is recycled back into cytoplasm. On the other hand, the exportins interact with their export cargoes in the nucleus only in presence of RanGTP and this trimeric complex leaves the nucleus through the NPC to deliver the exportin cargo to cytoplasm (reviewed in (14)).

Our unexpected finding that CC3 interacts with a number of importins β prompted us to examine what, if any, role CC3 might play in nuclear transport. The results of these studies clearly indicated

that CC3 acts to inhibit nuclear transport *in vitro* and *in vivo*.. Our work also showed that the pro-apoptotic activity of CC3 depends on its ability to inhibit nuclear import. This work is described in the manuscript that has been submitted for publication and is attached in Appendix to this report. Briefly, we have shown that CC3 interacts with importins β and also with the nuclear pore complex *in vitro and in vivo*. Importantly, unlike transport cargo proteins, CC3 interactions with karyopherins (and NPC) are not sensitive to RanGTP. Some of the cellular CC3 protein localizes at the NPC, and CC3 can "dock" importin β2 at the NPC. Excess of CC3 inhibits nuclear import of proteins with either M9 or NLS nuclear localization signal. The ability of CC3 to bind to both importins and NPC in Ran GTP independent manner is apparently key to its ability to inhibit nuclear import. Most importantly, inhibition of nuclear import is linked to pro-apoptotic activity of CC3.

Is CC3 protein involved in cellular stress responses? The obvious question that arises from our findings is why would cells need a protein that inhibits nuclear transport? We considered the possibility that CC3 might act to inhibit nuclear transport when cells are placed under stress. If so, cells with higher levels of CC3 might succumb to apoptosis when placed under particular types stress. Unfortunately, there are no reports in literature about how different types of stress influence nuclear transport except a single report that describes inhibition of nuclear transport by heat shock but not by osmotic or oxidative stress (13).

We reasoned that CC3 might play a role in heat shock responses in human cells by inhibiting nuclear transport. This possibility was supported by two unrelated observations: first, expression of CC3 was shown to be increased after heat shock treatment of head and neck carcinoma cells (10); second, nuclear import performed with permeabilized cells is inhibited at higher temperatures (unpublished). We have therefore decided to examine if higher levels of CC3 impair survival of cells after heat shock. Breast cancer cell lines were a clear choice to test this hypothesis because of the wide variation of CC3 expression levels in different lines, as well as availability of breast cancer cells modified by us to express ectopic CC3. The data in Figure 1 show a strong positive correlation between levels of CC3 and apoptotic responses to heat shock. These results, though limited to a few lines, show a strong correlation between heat shock induced cell death and levels of CC3 protein.

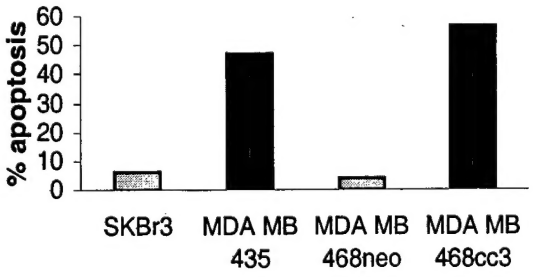

Figure 1. Cell lines SKBr3, MDA MB 435, MDA MB 468neo and MDA MB 468cc3 were subjected to heat shock at 44°C for 30 minutes and cultured for further 24 hrs, at which time they were harvested and processed for DNA content analysis by flow cytometric analysis of propidium iodide stained fixed cells. Percent apoptosis shows percent of cells with DNA content of less than 2n. Relative levels of CC3 expression are indicated below the chart.

The heat shock response is a highly conserved mechanism that protects cells against hyperthermia and other types of stress (6). Heat shock proteins (HSPs) are induced by stress and preserve structural integrity of the key regulatory proteins (6). HSPs are anti-apoptotic in context of a variety of death stimuli (reviewed in (17)). Hyperthermia (a procedure of raising the temperature of tumor bearing tissue to 40-42 degrees C) is used as an adjunctive therapy with chemo- and radio-therapy (CRT) (reviewed in (16)). Among other cancers, it is used with significant success for advanced primary and recurrent breast cancer (3). The mechanism of the beneficial effects of hyperthermia are poorly understood (5). Some evidence suggest that the responses of tumor cells versus normal cells to hyperthermia could be different (8). Even lacking understanding of its mechanisms, hyperthermia is

being pursued as a promising adjunctive therapy. We propose to focus our future research in analysis of the potential role of CC3 in responses of breast cancers to hyperthermia. If CC3 is indeed important in regulating these responses, it could be a unique predictive marker for adjunctive therapy with hyperthermia.

KEY RESEARCH ACCOMPLISHMENTS

- CC3 associates with five import receptors (importins β) and one export receptor (exportin 4).
- Interactions of CC3 with importins are not affected by RanGTP and its interactions with exportin do not depend on RanGTP. This proves that CC3 is not a "promiscuous" cargo of multiple karyopherins.
- CC3 localizes to a perinuclear area in the cells (identified by us recently as endoplasmic reticulum) and to the NPC.
- Purified CC3 protein inhibits nuclear import of substrates containing either classical NLS or the M9 type of nuclear targeting sequence in the assays of nuclear import *in vitro*.
- CC3 inhibits nuclear import by preventing nuclear translocation of importin β itself.
- The N terminal domain of CC3 binds to importins β and its C-terminus binds to NPC. CC3 therefore might inhibit nuclear import by "docking" importins at the nuclear pore and preventing their release by the nuclear RanGTP.
- Cells with higher levels of CC3 have lower rates of nuclear import, at least in the *in vitro* assay.
- A mutant CC3 lacking in ability to induce apoptosis is also incapable of inhibiting nuclear import.
- In microinjection experiments CC3, but not its mutant version, inhibits nuclear import *in vivo* and induces rapid cell death. These data show that the pro-apoptotic ability of CC3 depends on its ability to inhibit nuclear import.
- Higher levels of CC3 strongly predispose breast cancer to apoptosis induced by hyperthermia treatment

REPORTABLE OUTCOMES

A manuscript has been submitted for publication based on the research reported here (Appendix).
An application "The role of pro-apoptotic protein CC3 in regulation of stress responses in breast cancer cells" has been submitted to the DOD breast Cancer Research Program for funding as an IDEA award.

CONCLUSIONS

The finding that CC3 is likely to be involved in the regulation of nuclear transport is very important. Nuclear transport is an integral part of how cells regulate their growth and proliferation. Change in subcellular localization of a number of cellular proteins that perform key roles in various cellular processes is one of the ways through which cells regulate metabolic, proliferative and apoptotic responses. Therefore, identification of a normal cellular protein that can inhibit nuclear transport is a significant and novel finding. Importantly, we have obtained results that show that inhibition of nuclear transport by CC3 is tightly linked to its ability to induce apoptosis.
We propose that CC3 could act to inhibit nuclear transport under conditions of stress. Regulation of general nuclear transport by stress or other signals is an entirely unexplored area. Future work will concentrate on investigation of how CC3 links nuclear transport to stress responses such as apoptosis.

In particular, the role of CC3 in responses to treatment of breast cancer with hyperthermia (as adjunctive therapy) will be explored.

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Inhibition of Nuclear Import by CC3

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Running Title: CC3 inhibits nuclear import

We report here that normal cellular protein CC3, when in excess, inhibits nuclear import *in vitro* and *in vivo*. Recombinant CC3 binds directly to the transport receptors of the importin β family in a RanGTP-insensitive manner. Endogenous CC3 associates with importin β2/transportin and nucleoporins *in vivo*. In the *in vitro* nuclear import assays with permeabilized cells CC3 inhibits import of proteins possessing the classical nuclear localization signal (NLS) and the M9 signal. CC3 also inhibits nuclear translocation of transportin itself. Cells modified to express higher levels of CC3 have a slower rate of nuclear import, and, as described earlier, show an increased sensitivity to death signals. A mutant CC3 protein lacking this pro-apoptotic activity has a lower affinity for transportin, is displaced from it by RanGTP and fails to inhibit nuclear import *in vitro*. *In vivo*, microinjection of the wild type but not the mutant CC3 protein inhibits nuclear import and induces rapid cell death. Together, our results suggest that there is a direct correlation between the ability of CC3 to form a RanGTP resistant complex with importins, inhibit nuclear import and induce apoptosis.

Keywords: CC3/ transportin/ nuclear import/ apoptosis

Introduction

The bi-directional transport of macromolecules between the nucleus and cytoplasm through the nuclear pore complex (NPC) is an active and regulated process. It depends on carrier proteins known as importins and exportins (or karyopherins) that compose the importin β family with 22 members in humans (reviewed in (Strom and Weis, 2001). Members of this family can interact with their cargoes directly or use adapter proteins. Importin β1 mediates nuclear import of proteins containing a basic nuclear localization signal (NLS) by using importin α (Adam and Gerace, 1991; Gorlich et al., 1994; Weis et al., 1995). Using snurportin as an adapter, importin β imports small nuclear riboproteins (Palacios et al., 1997). Importin β2 also know as transportin, recognizes the M9-type nuclear localization sequences, and imports M9-containing RNA binding proteins without requiring an adapter molecule (Fridell et al., 1997; Nakielny et al., 1996; Pollard et al., 1996).

Importins β mediate interactions of transport complexes with the NPC. Importins β also bind to the small GTPase Ran that controls the directionality of the transport (reviewed in (Gorlich and Kutay, 1999; Kuersten et al., 2001)). Importins β bind specifically to the GTP loaded form of Ran found in the nucleus. The importin-cargo complex docks at the cytoplasmic face of the NPC, followed by the translocation across the NPC via interactions with nucleoporins (Ben-Efraim and Gerace, 2001). At the terminal nucleoporin, the cargo is released from importin into the nucleus by RanGTP. While RanGTP initiates the release of cargo from importins in the nucleus, exportins require RanGTP for binding to their various cargoes and subsequent nuclear export (reviewed in (Weis, 2002)).

Although the sequence similarity among importins β is low, they share conserved functional domains. Importins βcontain a N-terminal binding site for RanGTP and a NPC interacting domain. The latter mediates binding to the SLFG or FxFG repeats present in most nucleoporins (reviewed in (Ryan and Wente, 2000). The cargo-binding domain of importins β is located in the C-terminus (reviewed in (Strom and Weis, 2001). The common structural feature of importins is that they consist entirely of tandem HEAT (huntingtin-elongation A subunit-TOR) repeats. HEAT repeats form extended superhelical structures that are predicted to be involved in creation of protein recognition interfaces (Groves and Barford, 1999). Following RanGTP binding, the interconnected hairpin turns formed by HEAT repeats are thought to facilitate conformational changes in the structure of importin, which induces cargo release (Chook and Blobel, 1999).

Nucleocytoplasmic transport could be inhibited by interfering with the activity of proteins that mediate transport. Thus, inactivation of RanGTP (Izaurralde et al., 1997) or the addition of excess of nucleoporin binding domain of importin β (Kutay et al., 1997) block nuclear transport. Nuclear transport is also inhibited during infection by some lytic viruses (Chen et al., 1999; Dobbelstein et al., 1997; Gustin and Sarnow, 2001; Her et al., 1997). We report here that a cellular protein CC3 can inhibit nuclear import of substrates with different types of import signals. CC3 was first identified as a metastasis suppressor of the variant small cell lung carcinoma *in vivo* (Shtivelman, 1997). Its expression in tumor cells was shown to predispose them to apoptosis induced by a wide variety of death signals; acute overexpression of CC3 in cells induces cell death directly (Whitman et al., 2000). CC3 induced apoptosis does not appear to depend on p53 or involve classical downstream death responses, such as mitochondrial release of cytochrome c (Whitman et al., 2000), and its mechanism remains unclear. In this report, we demonstrate that the pro-apoptotic properties of CC3 might stem from its ability to inhibit the nuclear transport.

Results

Identification of proteins associated with CC3

To identify proteins that associate with CC3, purified GST-CC3 and GST were incubated with extracts prepared from MCF7 or HeLa cells metabolically labeled with ^{35}S-methionine. GST or GST-CC3 together with the bound cellular proteins were collected on glutathione-Sepharose, and bound proteins were resolved by SDS-PAGE. The same nine distinct protein bands were detected in a complex with GST-CC3 but not GST in both MCF7 and HeLa (Figure 1). When experiment was performed with larger amounts of cell extracts, bands were visible on Coomassie blue stained gels (data not shown). These protein bands were excised from the gel and subjected to analysis by mass spectrometry.

Positive identification was possible for proteins in seven out of nine bands, and a total of eight proteins were identified (one of the bands contained two individual proteins). Six of these were nuclear transport receptors of the importin β family (Figure 1): importin β_1, β_2 (transportin) and β_3 (importins 5, 7 and 9) and exportin-4 that mediates nuclear export of the translation initiation factor eIF-5A ((Lipowsky et al., 2000). Two other proteins were LRPPRC, also known as leucine rich protein-130 (DiSorbo et al., 1988), implicated in the nucleocytoplasmic shuttling of RNA (Liu and McKeehan, 2002; Mili et al., 2001) and human GCN, a homologue of yeast GCN1 that is necessary for activation of GCN2 kinase (Marton et al., 1993). All eight proteins found to bind to CC3 *in vitro* shared one common structural feature: they contain HEAT repeats. Importins β are entirely composed of 18-19 HEAT repeats (reviewed in (Strom and Weis, 2001); HEAT repeats are present in LRPPRC (Liu and McKeehan, 2002) and multiple HEAT repeats are found in the GCN1 sequence (data not shown).

CC3 associates with transportin and the nuclear pore complex in vitro and in vivo

We have examined interactions of CC3 protein with transportin (importin β_2 or MIP) *in vitro*. GST-CC3, when incubated with transportin polypeptides translated *in vitro*, bound to the full-length transportin and its C-terminal domain (amino acids 581-890) but not to the N-terminal amino acids 1-581 (Figure 2A). The N-terminus of importins β contains domains responsible for binding to RanGTP as well as to components of nuclear pore complex (Kutay et al., 1997). The *in vitro* translated N-terminal domain of transportin was functional because it could still bind to the Ran protein (Supplemental Figure 1).

Importantly, the association of CC3 and TRN was also observed between endogenous proteins *in vivo*. Transportin was immunoprecipitated from HeLa cells using specific antibodies, and CC3 was easily detected in complex with transportin (Figure 2 B).

We examined if, in addition to transport receptors, CC3 can associate with nuclear structures. An *in vitro* assay was developed to monitor interactions between whole nuclei and CC3. GST-CC3, GST-transportin (as a positive control) or GST (as a negative control) were incubated with purified nuclei isolated from cell line N417 that lacks endogenous CC3 (Shtivelman, 1997). Significant amounts of both GST-CC3 and GST-transportin but not GST were found associated with the pelleted nuclei (Figure 2C). This result suggests that CC3 can bind to some nuclear structures.

The subcellular localization of endogenous CC3 was analyzed by immunofluorescent staining. CC3 protein was found to localize in the perinuclear area and form a distinct ring at the perimeter of the nucleus (Figure 2D). Double staining with antibodies to CC3 and monoclonal antibody mAb414, which reacts with several FxFG-containing nucleoporins, showed a significant overlap of CC3 localization with the nuclear pore complex (Figure 2D).

To confirm the association of endogenous CC3 with the NPC by independent means we examined complex formation between CC3 and NPC *in vivo*. As shown in Figure 2E, CC3 was

detected in a complex with nucleoporins that were immunoprecipitated with mAb414. Only a fraction of cellular CC3 present in the extracts was immunoprecipitated by mAb414, as expected from the results of cell staining that shows the bulk of CC3 in the perinuclear structures (Figure 2D). We conclude that a fraction of cellular CC3 is found at the nuclear pore complex, though it remains to be determined if CC3 binds to NPC directly or through other proteins.

CC3 interactions with the NPC are independent of RanGTP

Interactions between CC3 and various karyopherins could indicate that CC3 is a "promiscuous cargo" or that it plays a role in nuclear transport. To distinguish between these possibilities, we examined if CC3 could be imported into nuclei *in vitro* and if the localization of CC3 is affected by RanGTP. We used the *in vitro* nuclear import assay (Adam *et al.*, 1990) with digitonin permeabilized HeLa cells in the presence and absence of exogenously added RanQ69L. RanQ69L is a Ran mutant that binds but does not hydrolyze GTP and remains in the GTP-bound form (Bischoff et al., 1994). RanGTP inhibits nuclear transport of multiple proteins by preventing their association with importins β (reviewed in (Kuersten et al., 2001; Macara, 2001; Weis, 2002)). Purified CC3 protein labeled with FITC was added to permeabilized HeLa cells in transport buffer or prepared cytosol (that contains nuclear transport factors). Under both conditions, CC3 localized at the nuclear envelope and in the perinuclear area of cytoplasm and was not imported into nuclei (Figure 3A). Addition of cytosol efficiently stimulated nuclear import of GST-M9, containing the M9 type of nuclear localization signal recognized by transportin (Nakielny et al., 1996), and M9 import was entirely inhibited by RanQ69L (Figure 3A). However, RanQ69L had no effect on localization of CC3 to the nuclear envelope (Figure 3A).

These results were confirmed using the nuclei sedimentation assay described above, where association of CC3 with the nuclei was not affected by addition of RanGTP (Supplemental Figure 2). Interaction of GST-transportin with the nuclei was diminished at higher concentrations of RanGTP as described (Ben-Efraim and Gerace, 2001). We conclude that CC3 is not imported into the nucleus, and binds to the NPC in a Ran-independent manner.

CC3 interactions with transportin and exportin 4 are independent of RanGTP

Having established that RanGTP has no effect on the interaction of CC3 with the nuclear envelope, we then examined how RanGTP might affect interactions between CC3 and karyopherins. Interactions of import receptors with their substrates are responsive to RanGTP, which triggers the dissociation of cargo from its receptor in the nucleus (Gorlich et al., 1996; Izaurralde et al., 1997; Rexach and Blobel, 1995). GST-CC3 was incubated with the *in vitro* translated transportin in the absence or presence of increasing amounts of RanGTP. RanGTP had little effect on the binding between transportin and GST-CC3 (Figure 3B), whereas it completely inhibited complex formation between transportin and its cargo GST-M9 (data not shown).

The effects of RanGTP on the association of CC3 with exportin 4 were also examined. Exportins require RanGTP for binding to nuclear substrates, which are subsequently exported to the cytoplasm (reviewed in (Gorlich and Kutay, 1999). However, GST-CC3 efficiently bound to exportin 4 in the absence of RanGTP (Figure 3C), and the addition of RanGTP had no effect on this binding. These results show that CC3 interactions with transportin and exportin 4 are independent of the Ran GTPase; therefore, CC3 is unlikely to be a cargo of either of these karyopherins.

CC3 competes with M9 substrate but not with RanQ69L for binding to transportin

We examined if CC3 interferes with the binding of substrates or RanGTP to transportin. Figure 3D shows that RanGTP efficiently binds to transportin even in presence of a large excess of CC3. However, binding of GST-M9, a transportin cargo, was inhibited when CC3 was present in excess (Figure 3E). It is unclear if M9 and CC3 compete for the same binding site on transportin or if binding of CC3 has an allosteric effect on transportin structure that inhibits subsequent binding of GST-M9.

CC3 inhibits nuclear import in vitro

We attempted to determine if CC3 might play a role in regulating nuclear transport when associated with importins and/or components of the NPC. The *in vitro* nuclear import assay was used to monitor effects of CC3 on the nuclear import of GST-M9, (a transportin cargo) and GST-GFP-NLS, which is imported by the complex of importins α and β_1. Both substrates were imported into the nucleus in presence of cytosol (Figure 4A). However, addition of purified CC3 to transport mixture resulted in a dramatic decrease of the nuclear import for both GST-M9 and GST-GFP-NLS. Partial inhibition was observed with CC3 concentrations as low as 1 μM (not shown), addition of 8 μM of CC3 resulted in apparently complete inhibition (Figure 4A). Inhibition of nuclear import by CC3 was not due to a nonspecific blocking of the NPC channel, because small proteins still could freely diffuse into nuclei in presence of 8 μM of CC3 (Supplemental Figure 3).

Using a different approach, the *in vitro* nuclear import assay was performed with cytosol prepared from either CC3-negative cells (N417neo) or from the same cells stably transfected to express high levels of exogenous CC3 (N417cc3) (Shtivelman, 1997). As described previously (Shtivelman, 1997; Whitman et al., 2000), the N417cc3 cells have a relatively high rate of spontaneous apoptosis under normal conditions and are much more sensitive to a variety of death signals compared to control N417neo clones. We have estimated the intracellular concentration of exogenously expressed CC3 in N417-CC3 cells to be around 9 μM. A significant amount of CC3 protein from N417-CC3 cells is found in the cytosol fraction that was prepared for the *in vitro* import assay (data not shown). GST-M9 or GST-GFP-NLS was added to permeabilized cells in the presence of cytosol from N417neo or N417cc3 cells. The addition of cytosol from N417-neo cells, similar to HeLa cytosol, fully supported the nuclear import of both GST-M9 and GST-GFP-NLS proteins (Figure 4B). Same concentration of cytosol from N417cc3 cells failed to support nuclear import of either substrate. We conclude that high levels of CC3, either recombinant or cellular, inhibit nuclear import.

High levels of cellular CC3 in permeabilized cells diminish the rate of nuclear import in vitro

The results described above suggest that the rate of nuclear import could be unusually rapid in cells where CC3 is absent. We have therefore performed *in vitro* import assays with N417 clones described above. Because these cells grow in suspension, the assays were conducted in suspension and analyzed by FACS (fluorescence activated cell sorting) as described (Paschal et al., 1996). We found that while import of GST-M9 in N417cc3 cells was insignificant for the first 20 minutes, in N417neo cells it has reached its maximum level at this time (Figure 4C and D).

We have also compared the rates of nuclear import in clones derived from a melanoma line C32r. The expression level of endogenous CC3 in C32r cells is extremely low, with intracellular concentration well below 0.1 μM (not shown). A high level of expression of exogenous CC3 was introduced into these cells (NicAmhlaoibh and Shtivelman, 2001). *In vitro* import assays showed practically no nuclear fluorescence after a 5 minute incubation in C32cc3 cells, while C32neo cells showed a robust nuclear import at this time (Figure 4E). Only after 20 – 30

minutes of incubation the amount of imported substrate in C32cc3 cells reached levels seen in C32neo cells (Figure 4E). These data show that CC3 remaining in the cells after mild extraction (data not shown) is capable of slowing down the rate of nuclear import and strongly indicate that cells with different concentrations of cellular CC3 might have different rates of nuclear import in *vivo*.

CC3 inhibits nuclear translocation of transportin

Effect of CC3 on nuclear translocation of transportin was examined in permeabilized cells. Transportin alone, as shown previously (Nakielny and Dreyfuss, 1998), translocates into the nuclei and is not retained in remaining cytosolic structures (Figure 4F, panel 1). However, in the presence of even relatively low concentrations of CC3, the nuclear translocation of transportin was diminished and much of it was "docked" at the nuclear envelope and in the extranuclear structures (Figure 4F, panel 2). Accumulation of transportin in cytoplasmic structures was time-dependent (Supplemental Figure 4), and more pronounced at higher concentrations of CC3 (Figure 4F), when localization of transportin resembled the distribution of CC3 protein itself in permeabilized cells (Figure 3A). These results suggest that CC3-mediated "docking" of importins at the NPC and in the cytoplasm could be involved in the negative regulation of nuclear transport by CC3.

CC3 interacts with transportin through its N-terminus and with the nuclear envelope through its C-terminus

Mutant and truncated forms of CC3, which differ in their ability to induce apoptosis, were used to examine in more detail the binding of CC3 to importins and the NPC (Figure 5A). The N-terminal 94 amino acids of CC3 (CC3-N) were shown to be sufficient to induce apoptosis (Whitman et al., 2000). We have also prepared expression constructs for the C-terminal domain of CC3 (amino acids 100-242) and a mutant version of CC3. The design of mutant CC3, where glycines 28 and 31 were substituted with valine and alanine, respectively, was based on a published report (Xiao et al., 2000). These mutations have been shown to inhibit the proapoptotic properties of CC3 ((Xiao et al., 2000) and our unpublished results). We examined the cellular localization of these polypeptides in transiently transfected HeLa cells. Unlike wild type CC3 protein that localized to the perinuclear areas and nuclear envelope, mutant CC3 was also detected diffusely in the entire cytoplasm and within the nucleus itself (Figure 5B). Most of CC3-N polypeptide localized in the nucleus and some was seen diffusely throughout the cytoplasm, but it was not detected at the nuclear envelope. The C-terminus of CC3, however, retained the ability to associate with the nuclear envelope and with perinuclear structures (Figure 5B). Identical results were obtained with CC3 polypeptides transiently expressed in HeLa cells as fusions with GFP protein (data not shown). These results show that binding of CC3 to the nuclear envelope is mediated through sequences in its C-terminal domain.

Next, we examined which domain of CC3 is responsible for its association with transportin *in vitro*. Both wild type and mutant CC3 were detected in a complex with GST-transportin (Figure 5C). The N-terminal domain displayed a much higher affinity for GST-transportin as compared to the full length CC3. However, the C-terminus was not detected in a complex with GST-transportin (Figure 5C). We conclude that the amino-terminus of CC3 mediates binding to importins while the carboxyl-terminus associates with the nuclear envelope. Therefore, we suggest that CC3 might associate with the nuclear pore complex both directly and indirectly through its interactions with import receptors.

A mutant CC3 protein lacking proapoptotic properties shows weakened interactions with both transportin and NPC.

To examine possible reasons for the different localizations of the mutant and wild-type CC3, we analyzed interaction of mutant CC3 with the NPC, and if it could be imported into the nuclei *in vitro*. We found that recombinant mutant CC3, when added in transport buffer to permeabilized HeLa cells, showed some association with the nuclear envelope, but was strongly retained all throughout remaining extranuclear structures (Figure 6A, panels 1). Importantly, as seen with the transfected mutant CC3 (Figure 5B), recombinant mutant CC3 was also found within the nuclei (Figure 6A). Addition of increasing amounts of RanGTP to permeabilized cells inhibited the association of mutant, but not wild-type CC3 with the nuclear envelope. At higher concentrations of RanGTP mutant CC3 failed to associate with the NPC or to be imported into nuclei (Figure 6A). Because these experiments were conducted in transport buffer alone, we conclude that the mutant CC3 has a weakened ability to interact with the NPC and is vulnerable to dissociation by excess RanGTP.

Differences in the affinities of wild type and mutant CC3 for the NPC could be secondary to their different affinities for importins. To address this possibility, wild type and mutant GST-CC3 proteins were incubated with the *in vitro* translated transportin in presence of increasing amounts of RanGTP. Mutant CC3 indeed displayed a weaker affinity for transportin as compared to wild type proteins under the same conditions (Figure 6B, lane 1). More importantly, RanGTP was able to effectively inhibit binding of transportin to the mutant CC3 (Figure 6B, lanes 2-4). These results show that mutant CC3 is vulnerable to RanGTP mediated dissociation from transportin. Together with the observed partial nuclear import of mutant CC3, these results suggest that, unlike the wild type protein, mutant CC3 might behave more like a transportin cargo.

CC3 mutant lacking proapoptotic properties does not inhibit nuclear import in vitro or in vivo

We examined if the mutant CC3 protein lacking pro-apoptotic activity can inhibit nuclear import of substrates in the *in vitro* import assay. In vitro import assay with GST-M9 or GST-GFP-NLS was performed in presence of increasing amounts of mutant CC3. Unlike wild-type CC3 (Figure 4A), mutant CC3 had no effect on the nuclear import of either the GST-M9 or GST-GFP-NLS cargo proteins (Figure 7A). Together, these results suggest that the inhibition of nuclear import by wild type CC3 is directly related to its ability to form a RanGTP resistant complex with importins. The pro-apoptotic ability of CC3 proteins therefore appears to be related to their ability to inhibit nuclear import.

To strengthen the connection between inhibition of nuclear transport and pro-apoptotic characteristics, the wild type and mutant CC3 proteins were injected into the cytoplasm of live NIH3T3 cells together with the GST-GFP-NLS. As expected, GST-GFP-NLS alone (or co-injected with GST) assumed nuclear localization within 15 minutes after injection. Mutant CC3 had no effect on the localization of GST-GFP-NLS (Figure 7B). However, the co-injection of wild-type CC3 led to a dramatic retention of substrate in the cytoplasm (Figure 7B). At thirty minutes after co-injection of wild type CC3 and GST-GFP-NLS, the majority of cells had very little nuclear GFP, and many cells assumed a rounded morphology (not shown). Within 2 hours of injection, 70 to 80 % of GFP-positive cells injected with wild-type CC3 had floated up from the surface of the dish. At this time, at least 80-90 % of cells injected with GST-GFP-NLS alone or together with mutant CC3 remained alive as judged by their morphological appearance (data not shown). We conclude that there is a direct relationship between the ability of CC3 to inhibit nuclear import and induce cell death.

Discussion

This work was initiated in order to elucidate the mechanism through which CC3, when overexpressed in tumor cells, induces apoptosis. CC3 was initially identified as a gene whose expression is absent in metastatic small cell lung carcinoma (SCLC) cells and whose ectopic suppressed metastasis (Shtivelman, 1997). The metastasis-suppressing ability of CC3 was linked to its pro-apoptotic characteristics: SCLC cells forced to express CC3 showed a high rate of spontaneous apoptosis under normal growth conditions and became highly sensitive to a variety of death signals that they would otherwise resist (Shtivelman, 1997). Moreover, acute overexpression of CC3 directly induces cell death in transiently transfected cells (Whitman et al., 2000). However, the mechanisms underlying the pro-apoptotic activity of CC3 remained a mystery. The sequence of CC3 did not provide any clues in this regard, even though significant sequence homology was reported between CC3 and the members of a large family of short-chain dehydrogenases-reductases or SDRs (Baker, 1999). CC3 was subsequently identified as a HIV Tat binding protein, named TIP30, and shown to stimulate Tat-induced transcription (Xiao et al., 1998). Xiao et al later reported that CC3 has a kinase activity, and suggested that the residues 22-35 comprise the ATP-binding domain of CC3/TIP30 kinase (Xiao et al., 2000). This same domain, well conserved between CC3 and a variety of SDR enzymes, was predicted and modeled to serve as a NADP(H) binding site (Baker et al., 2000). In order to understand the possible biochemical function of CC3 we have decided to characterize protein interactions involving this protein.

Unexpectedly, we have found that CC3 interacts with six members of the importin β family, including five importins and an exportin and with two additional cellular proteins that, like importins, contain HEAT repeats. However, CC3 is not a "promiscuous" cargo protein that uses different transport receptors as has been shown for some ribosomal proteins and the signal recognition particle SRP19 (Dean et al., 2001; Jakel and Gorlich, 1998; Rout et al., 1997). Interactions of CC3 with transportin and exportin 4, unlik einteractions of cargo proteins, are not regulated by RanGTP. In addition, the observed association of CC3 with the NPC and its physical interactions with FXFG repeat containing nucleoporins *in vivo* argue against the possibility that CC3 is simply a cargo protein of several importins β. Most importantly, we show that excess of CC3 inhibits nuclear import *in vitro* and *in vivo*. A clue to the mechanism of this inhibition might be found in the demonstrated here ability of CC3 to associate *in vivo* not only with importins but with the nuclear pore complex as well.

CC3 appears to be a unique protein capable of binding to both importins and nucleoporins in a Ran-independent manner. SUMO-1 deconjugating protein of yeast, Ulp1 is the only other non-cargo protein that associates with three yeast karyopherins in a Ran-independent manner (Panse et al., 2003). According to different studies, Ulp1 might bind to nucleoporins directly (Takahashi et al., 2000), or indirectly through RanGTP-insensitive interactions with karyopherins (Panse et al., 2003). The human homologue of Ulp1, SENP2, was shown to interact with Nup153 directly and is retained at the NPC (Hang and Dasso, 2002; Zhang et al., 2002). The domain of SENP2 responsible for its localization at the NPC was mapped to the first 63 amino acids of its sequence (Hang and Dasso, 2002; Zhang et al., 2002). We have noticed a limited homology between amino acid residues 4-30 of SENP2 and the amino acids 182-209 in the C-terminal domain of CC3, with 15 identical amino acids residues and 3 conserved substitutions (Figure 7C). It would be of interest to examine the functional significance of this sequence similarity and to determine if interaction of CC3 with the NPC depends on its association with importins.

Our results show that excess of CC3 inhibits nuclear import of proteins with either a classical NLS or M9 type import signal, indicating that CC3 could inhibit transport mediated by transportin (M9) and by importin α: β complexes as well. It is likely that the mechanism of inhibition is related to the ability of CC3 to inhibit the nuclear translocation of transportin itself (and probably other importins) (Figure 4F). It is also possible that the ability of CC3 to compete with cargo proteins for binding to transportin (Figure 3E) contributes to the inhibition of nuclear import by CC3, especially if the affinity of CC3 for importins is higher than that of regular cargo proteins. Indeed, in the *in vitro* import assay CC3 apparently is able to tether transportin to the remaining extranuclear structures (Figure 4F), indicating a relative strong association between the two.

We suggest, however, that the key to the inhibitory effect of CC3 on nuclear import lies in our findings that its interactions with karyopherins and nuclei are insensitive to RanGTP. As seen in Figure 4F, excess of CC3 leads to retention of transportin in extranuclear structures and at the nuclear envelope. Insensitivity to RanGTP would have profound consequences for the fate of transportin-CC3 complexes interacting with the NPC. We have no evidence to suggest that transportin bound to CC3 cannot translocate through the NPC channel, though it must remain a possibility at this time. However, if translocation of the transportin-CC3 complex through the NPC channel indeed occurs, this complex would remain bound to the nucleoplasmic side of the NPC. Even though binding of transportin to the NPC on the nucleoplasmic side would be disrupted by RanGTP, binding of CC3 to the NPC would not be affected. This could leave transportin tethered to the NPC through the C-terminal domain of CC3. Transportin (and possibly other importins), thus immobilized, would be unable to return to the cytoplasm and continue the nuclear transport functions. Not only the turn-around of karyopherins at the NPC would be affected but also many of their binding sites on the NPC would remain unproductively occupied therefore limiting the rate of nuclear transport.

By demonstrating that a mutant CC3, deficient in apoptotic activity, does not inhibit nuclear transport, this study has established a strong correlation between the pro-apoptotic potential of CC3 and its negative effect on the nuclear transport. Indeed, the injection of wild type CC3 into live cells not only inhibits nuclear import of a NLS containing substrate but also induces rapid cell death, presumably by disrupting the nuclear transport pathway in general. Mutant CC3 does not inhibit nuclear import, nor does it induce cell death. The vulnerability of mutant CC3-importin complexes to dissociation by RanGTP may underlie the inability of the mutant CC3 to inhibit nuclear import and induce cell death.

The need for a cellular protein capable of inhibiting nuclear import is not immediately clear. We believe that under certain conditions cells might have to inhibit nuclear transport. There are some published evidence to support this. The classical nuclear import pathway and export of poly(A) RNA export is inhibited by heat shock (Liu et al., 1996). In addition to heat shock, several other types of stress in S. cerevisiae also inhibit protein import and that involves redistribution of the yeast homologue of RanGTP in the cytoplasm (Stochaj et al., 2000). Nuclear import has been shown to be downregulated by phosphorylation (Kehlenbach and Gerace, 2000). Reduction in the rate of nuclear import was shown to occur in aged fibroblasts (Pujol et al., 2002) suggesting that cells indeed possess mechanisms to negatively regulate nuclear transport. We speculate that during stressful conditions the levels of CC3 protein could be elevated or it could be recruited to the nuclear envelope resulting in reduced efficiency of nuclear transport. Indeed, the expression of CC3 was reported to be significantly increased after heat-shock treatment (Narita et al., 2002). Under normal conditions, the inhibitory activity of endogenous CC3 could be subject to additional negative regulation, for example, by

sequestration of the bulk of cellular CC3 protein in extranuclear compartments away from the NPC.

Nuclear transport has been shown to be affected early in apoptosis, and the re-distribution of transport factors occurs prior to the collapse of the nuclear permeability barrier (Buendia et al., 1999) (Ferrando-May et al., 2001) The lack of CC3 expression in tumors with aggressive and metastatic character might indicate a selective advantage for CC3-negative cells in resisting potentially apoptogenic stress signals. Obviously, further work will be needed to fully understand the significance of CC3-induced inhibition of the nuclear import pathway.

Materials and Methods

Identification of CC3-binding proteins

Hela or MCF7 cells were metabolically labeled with [^{35}S] methionine and lysed in buffer A containing 25 mM Tris pH 7.5, 0.5% Triton X-100, 150 mM NaCl and Complete Protease Inhibitors (Roche). GST or GST-CC3 was added to the clarified lysate, and the samples were diluted 5-fold with buffer B (25 mM Tris pH 7.5, 0.1% Triton X-100, 100 mM NaCl and protease inhibitors), followed by shaking at 4°C for 1 hour. Proteins bound to GST-CC3 were recovered with glutathione-Sepharose, and resolved on 5% SDS-PAGE. The bound proteins were detected by autoradiography, or silver staining, or Coomassie Blue staining. Proteins bands associated with GST-CC3 were excised from the gel and subjected to MALDI-Mass Spectrometry analysis and identification at the Protein Chemistry Core Facility, Howard Hughes Medical Institute, Columbia University, New York.

Protein expression and purification

GST and His-tag fusion proteins were expressed in bacteria under standard conditions. GST fusion proteins were purified by affinity chromatography on glutathione-Sepharose beads (Amersham Biotechnology) and eluted from the beads with 20 mM reduced glutathione or by cleavage with thrombin. His-tagged proteins were first purified on Ni-NTA agarose according to manufacture's protocol (Qiagen), followed by passage through a Superose 12 gel exclusion column (Amersham Biotechnology) in column buffer (25 mM HEPES pH 7.6, 1 mM DTT, 5% glycerol and 150 mM NaCl) and concentrated with a Centricon-10 (Millipore). Coupled *in vitro* transcription - translation of proteins was done using wheat germ extracts in the presence of [^{35}S] methionine according to the manufacturer's protocol (Promega).

Nuclei sedimentation assays

Small lung cell carcinoma cells N417 were lysed in hypotonic buffer (10 mM Tris pH 7.5, 1.5 mM $MgCl_2$, 10 mM NaCl with protease inhibitors) by Dounce homogenization. Nuclei were separated from the cytosol fraction by centrifugation at 1,000 g for 3 minutes, washed 2 times with buffer C (25 mM HEPES pH 7.6, 1 mM DTT, 100 mM NaCl and protease inhibitors) and stored in buffer C with 50% glycerol at -80°C. For the nuclei sedimentation assay recombinant proteins at 1 μM were mixed with purified nuclei (equivalent of 25 μg of protein) in a final volume of 50 μl in buffer D (25 mM HEPES pH 7.6, 1 mM DTT, 5% glycerol, 100 mM KCl, 5 mM $MgCl_2$, 1 mg/ml BSA, 0.5 mM ATP, 0.5 mM GTP, 10 mM phosphocreatine and 20 units/ml creatine kinase) and incubated for 15 minutes at room temperature with shaking. Nuclei were pelleted at 1,000 g for 3 minutes, resuspended in 20 μl buffer D, layered onto 100 μl 25% sucrose in buffer D (without BSA) and sedimented for 15 minutes at 20,000 g. The pellets containing nuclei were resuspended in SDS sample buffer for PAGE analysis.

Antibodies to CC3

Polyclonal antisera to CC3 was raised in rabbits using purified recombinant GST-CC3 as an antigen. Immune serum was purified first on column of immobilized GST protein to remove the GST-reactive antibodies followed by affinity purification on a column of immobilized CC3 protein.

Protein labeling with FITC

5-10 mg/ml GST-M9 or GST-CC3 was dialyzed in 1L labeling buffer (25 mM HEPES pH 7.6, 5% glycerol and 150 mM NaCl). Fluorescein-5-maleimide (Molecular Probes) was added to achieve a 2:1 molar ratio of fluorescein to protein, and samples were incubated for one hour on ice. Non-bound fluorescein was removed with a NAP-5 column (Amersham Biotechnology) followed by concentration with a Centricon-10 spin column.

Co-immunoprecipitation

HeLa cell lysates for immunoprecipitation were prepared as described (Xu et al., 2002). Briefly, cell extracts were prepared by sonicating and subjected to immunoprecipitation with mAb414 (Covance) or anti-transportin (Transduction Labs) mouse monoclonal antibodies. The immune complexes were collected on protein G-agarose (Invitrogen) and analyzed by Western blotting with relevant antibodies.

In vitro nuclear import assays

The *in vitro* nuclear import assays were performed according to the published protocol (Adam et al., 1990). Hela cells grown on cover slips were washed with PBS and permeabilized with 30 μg/ml digitonin in buffer E (25 mM HEPES pH 7.6, 1 mM DTT, 5% sucrose, 110 mM KH_3CO_2, 5 mM MgH_3CO_2 and 2.5 mg/ml BSA) for 5 minutes at room temperature. Permeabilized cells were washed four times with buffer E and inverted onto 15 μl of import mixture containing the indicated proteins added with or without cytosol from Hela cells in transport buffer (buffer E + 0.5 mM ATP, 0.5 mM GTP, 10 mM phosphocreatine and 20 units/ml creatine kinase). Coverslips were incubated 20 minutes at room temperature. Reactions were stopped with 2% paraformaldehyde for 20 minutes at room temperature. When cells were further processed for immunofluorescence, they were permeabilized with 0.2% Triton X-100 in PBS for 10 minutes at room temperature prior to antibody staining.

GST pull-down assays

GST or GST fusion proteins at concentration of 10 μM were mixed for 1 hour at 4°C with 7.5 μl (bed volume) of glutathione-Sepharose in a final volume of 20 μl in buffer F (25 mM HEPES pH 7.6, 1 mM DTT, 5% glycerol, 100 mM KCl and 0.1% Triton X-100). Glutathione-Sepharose beads with bound GST fusion proteins were washed with reaction buffer (buffer F + 5 mM $MgCl_2$, 1 mg/ml BSA and protease inhibitors) and mixed with 10 μl of the *in vitro* translated proteins in a final volume of 100 μl in the reaction buffer and incubated for 1 hour at 4°C. Where indicated, His-RanQ69L or CC3 were added at 1, 4 or 8 μM. When RanQ69L was added, reactions also contained 1 mM GTP, 10 mM phosphocreatine and 20 units/ml creatine kinase. Glutathione-Sepharose beads were washed with buffer F and resuspended in the SDS sample buffer for gel analysis. Pull-down assays with Ni-NTA agarose (Qiagen) were performed in a similar manner, except the buffers contained 0.5 % Triton X-100, 10 % glycerol and 5 mg/ml BSA.

Microinjections

3T3 cells grown in DMEM + 10% FBS on 4 cm glass bottom plates (MatTek Corporation) were microinjected into the cytoplasm with the indicated recombinant proteins using Eppendorf Transjector 5246, followed by incubation at 37°C. Protein localization of live or fixed cells was determined by fluorescent microscopy.

Acknowledgements

We thank Dr. Brian Cullen for the gift of transportin cDNA, Dr. Gideon Dreyfuss for GST-M9 and GST-transportin constructs, Dr. Dirk Gorlich for His-exportin 4 plasmid, Dr. Ian Macara for the GST-GFP-NLS plasmid, and Dr. Karsten Weis for RanQ96L. We are grateful to Dr. Andy Finch for his guidance in the microinjection experiments. This work was supported by the grant DAMD17-01-1-0191 from USAMRAA and by institutional funds to E. S.

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Legends to Figures.

Figure 1. Identification of proteins associated with CC3. Hela cells were metabolically labeled with [^{35}S] methionine and lysed. Cytosolic protein extracts were incubated with GST or GST-CC3. Bound proteins were recovered on glutathione-Sepharose, resolved by SDS-PAGE and detected by autoradiography.

Figure 2. CC3 associates with transportin and the NPC *in vitro and in vivo.*
(**A**) *In vitro* translated transportin polypeptides: full-length (FL), N-terminal amino acids 1-581 (TRN-N) and C-terminal amino acids 581-890 (TRN-C) were incubated with GST or GST-CC3. Complexes were recovered with glutathione-Sepharose, washed, resolved by SDS-PAGE and detected by autoradiography. (**B**) HeLa cells extracts were subjected to immunoprecipitation with a control IgG or anti-transportin antibody. The immune complexes were resolved by electrophoresis and analyzed by Western blotting with antibodies to transportin (TRN) and CC3. (**C**) Nuclei purified from N417 cells were incubated with GST, GST-CC3 or GST-TRN. Proteins bound to nuclei after sedimentation as described in Materials and Methods were resolved by PAGE and detected by western blotting with anti-GST polyclonal antibodies. (**D**) Hela cells were processed for immunofluorescence with the anti-CC3 rabbit polyclonal antibody and anti-NPC mouse monoclonal mAb414 (Covance), followed by detection with anti-rabbit IgG-CY3 (red) and anti-mouse IgG-FITC (green), and analyzed by confocal microscopy. (**E**) mAb414 was used to immunoprecipitate components of the NPC from HeLa cells extracts as described in Materials and Methods. The immune complexes were analyzed by Western blotting with mAb414 and antibodies to CC3.

Figure 3. Interactions of CC3 with NPC, transportin and exportin 4 are resistant to dissociation by RanGTP. (**A**) FITC-labeled GST-M9 or GST-CC3 at 0.5 μM were added to permeabilized Hela cells in transport buffer with or without 50 μg Hela cytosolic extract and 8 μM RanQ69L. After incubation for 20 minutes at room temperature cells were fixed and examined by fluorescent microscopy. (**B**) *In vitro* translated transportin (lane 1) was incubated with GST-CC3 in the absence (lane 2) or in the presence of 1, 4 and 8 μM RanQ69L (lanes 3-5). Complexes were recovered with glutathione-Sepharose, resolved by SDS-PAGE and detected by autoradiography. (**C**) His-exportin-4 at 5 μM was incubated with GST (lane 1) or GST-CC3 without RanQ69L (lane 2) or in the presence of 2.5, 5 and 10 μM RanQ69L (lanes 3-5) for 1 hour at 4°C. Complexes were recovered with glutathione-Sepharose, resolved by SDS-PAGE and detected by staining with Coomassie Blue. (**D**) *In vitro* translated transportin was incubated with His-RanQ69L in the absence (lane 3) or presence of 1, 4 and 8 μM GST-CC3 (lanes 4-6). Complexes were recovered with Ni-NTA agarose,and analyzed by SDS-PAGE followed by autoradiography. Lane 2 shows the amount of transportin that was incubated without RanQ69L and bound to the Ni-NTA agarose in a non-specific manner. (**E**) *In vitro* translated transportin was incubated with GST-M9 in absence (lane 2) or in presence of 1, 4 and 8 μM CC3 (lanes 3-5). Complexes were analyzed as described in (B).

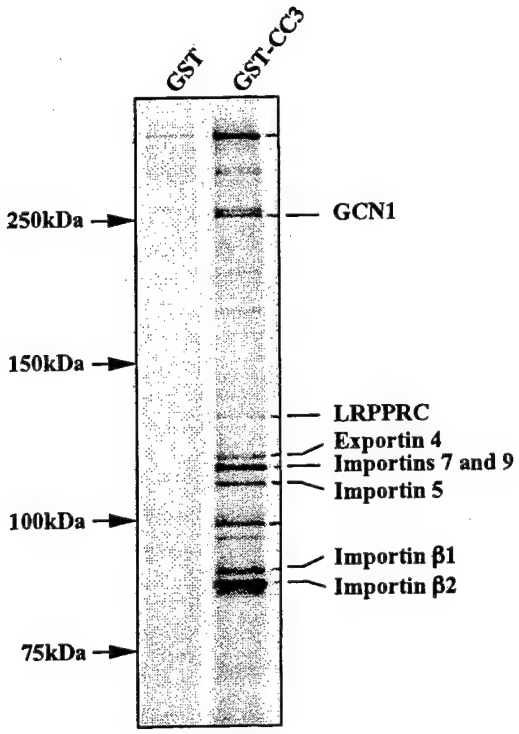
Figure 4. CC3 inhibits the nuclear import of substrates containing NLS or M9 import signals and nuclear translocation of transportin. (**A**) FITC-labeled GST-M9 at 0.5 μM or GST-GFP-NLS (1 μM) was added to permeabilized Hela cells in transport buffer with or without 50 μg Hela cytosolic extract and 8 μM CC3. After incubation for 20 minutes at room temperature, cells were fixed, and the localization of import cargoes was examined by fluorescent microscopy. (**B**) FITC-GST-M9 or GST-GFP-NLS was added to permeabilized Hela cells in transport buffer or

with 150 μg cytosolic extract prepared from either CC3-negative cells (N417neo) or from the same cells stably transfected to express high levels of CC3 (N417cc3). Cells were processed as in (A). (**C**) *In vitro* import of FITC-GST-M9 (0.5 μM) was performed in suspension with N417neo and N417cc3 cells and the nuclear fluorescence of was analyzed by FACS. FL1 fluorescence histograms of live cells are shown for cells incubated for 30 mins with GST-M9 but without cytosol (no cytosol) and for cells incubated with both GST-M9 in cytosol for 10 min. (**D**) Graphic representation of the results of the *in vitro* import assays with N417 clones performed as described in (C). Data are shown as relative fluorescence where the fluorescence of cells incubated with substrate in the absence of cytosol is assigned an arbitrary value of 1. The experiment was performed twice with nearly identical results. (**E**) FITC-labeled GST-M9 (0.5 μM) was added to permeabilized C32neo and C32cc3 cells in transport buffer with 50 μg of cytosolic extract prepared from N417neo cells. Cells were processed as in (A) after different times of incubation (**F**) GST-transportin at 0.5 μM was added to permeabilized Hela cells in transport buffer alone (panel 1) or in the presence of 2, 4, 8 μM CC3 (panels 2-4). After incubation for 20 minutes at room temperature cells were fixed and permeabilized with Triton X-100. The cellular localization of transportin was examined by immunofluorescent microscopy using polyclonal anti-GST antibodies and anti-rabbit IgG-FITC.

Figure 5. CC3 interacts with transportin through its N-terminus and with the NPC through its C-terminus. (**A**) Schematic representation of CC3 constructs used and their pro-apoptotic activity. (**B**) N-terminally Flag tagged expression constructs for WT, CC3-N, CC3-C and mutant CC3 were transiently transfected into Hela cells. Cells were fixed 24 hours later and examined by immunofluorescent confocal microscopy with anti-epitope tag antibodies. (**C**) *In vitro* translated wild type and mutant CC3, CC3-N and CC3-C polypeptides were incubated with GST or GST-transportin. Complexes were recovered with glutathione-Sepharose, resolved by SDS-PAGE and bound ^{35}S-methionine labeled proteins was detected by autoradiography.

Figure 6. Interactions of mutant CC3 with transportin and the NPC are vulnerable to dissociation by RanGTP. (**A**) GST-CC3 or GST-mutant CC3 at 0.5 μM was added to permeabilized Hela cells in transport buffer without (panels 1) or with 1, 4, or 8 μM RanQ69L (panels 2-4). After incubation for 20 minutes at room temperature, cells were examined by immunofluorescent microscopy with anti-GST polyclonal antibodies. (**B**) *In vitro* translated transportin was incubated with GST-CC3 or GST-mutant CC3 in the presence of 1, 4 and 8 μM RanQ69L. Presence of TRN in complexes was detected by autoradiography.

Fig. 7. Lack of apoptotic activity of mutant CC3 correlates with the loss of ability to inhibit nuclear import *in vitro* and *in vivo*. (**A**) GST-M9-FITC (0.5 μM) or GST-GFP-NLS (1 μM) was added to permeabilized Hela cells in transport buffer with 50 μg of Hela cytosolic extracts without (panels 1) or with 2, 4 or 8 μM of mutant CC3 protein (panels 2-4). After incubation for 20 minutes at room temperature, cells were fixed, and the cellular localization of proteins was examined by fluorescent microscopy. (**B**) GST-GFP-NLS (0.5 mg/ml) was injected into cytoplasm of NIH3T3 cells with GST-CC3 (5 mg/ml) or GST-mutant CC3 (5 mg/ml), followed by incubation for 30 minutes at 37°C. Cells were fixed and protein localization was examined by fluorescent microscopy. (**C**) Sequence alignment of CC3 and human SENP2.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GST-CC3
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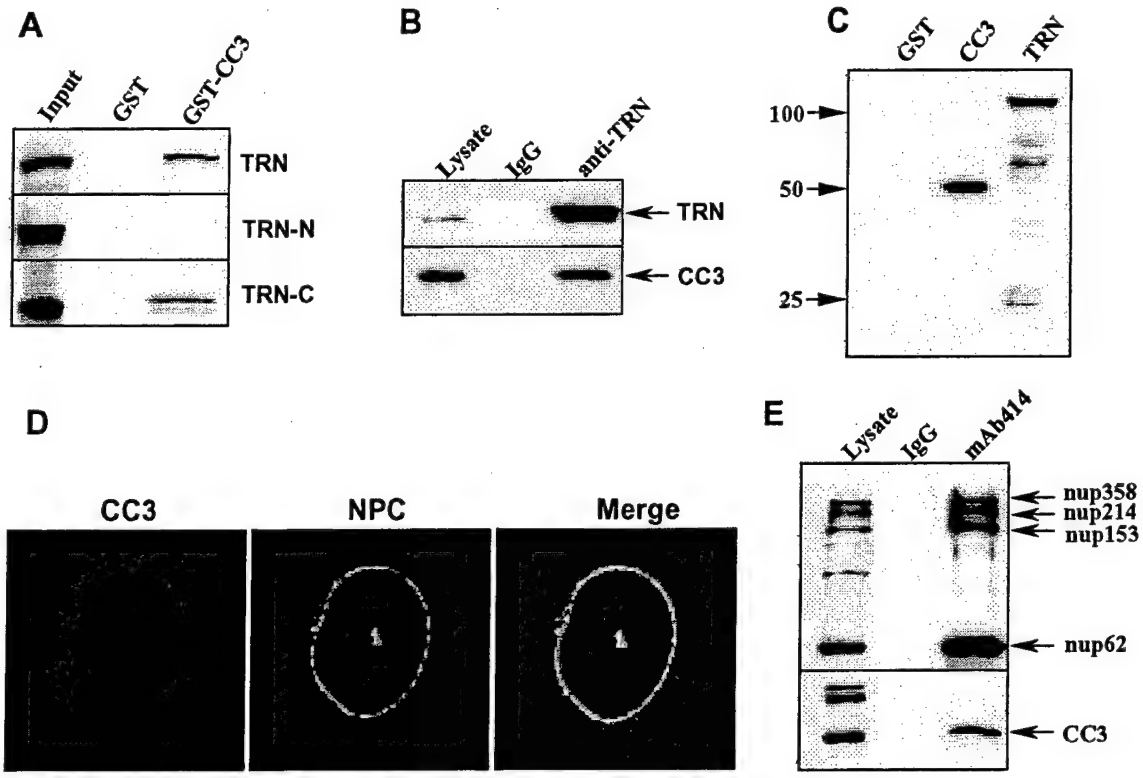
75kDa
GCN1
LRPPRC
Exportin 4
Importins 7 and 9
Importin 5
Importin β1
Importin β2

Figure 1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GST-CC3
TRN
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TRN-C
B
Lysate
IgG
anti-TRN
TRN
CC3
C
GST
CC3
TRN
100
50
25
D
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Merge
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IgG
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nup358
nup214
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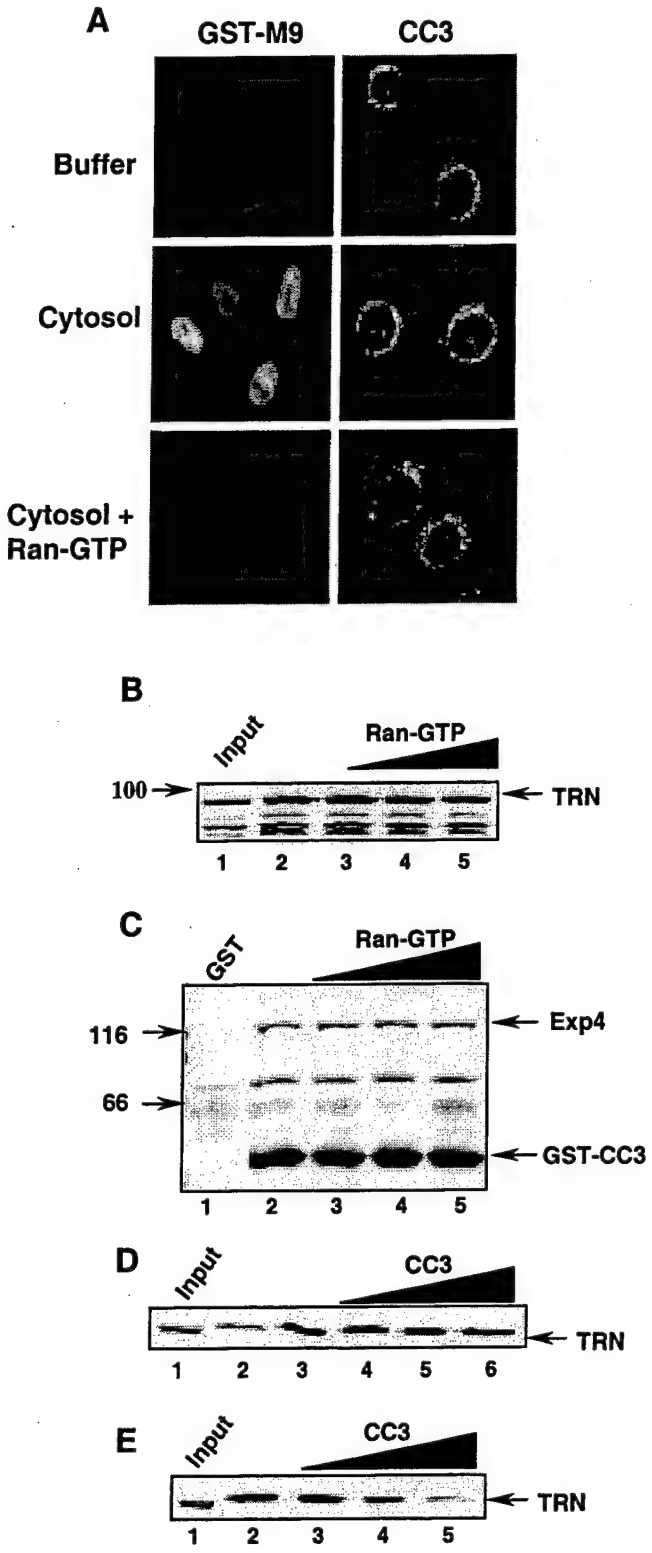
nup62
CC3

Figure 2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CC3
Buffer
Cytosol
Cytosol +
Ran-GTP
B
Input
Ran-GTP
100
TRN
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C
GST
Ran-GTP
116
66
Exp4
GST-CC3
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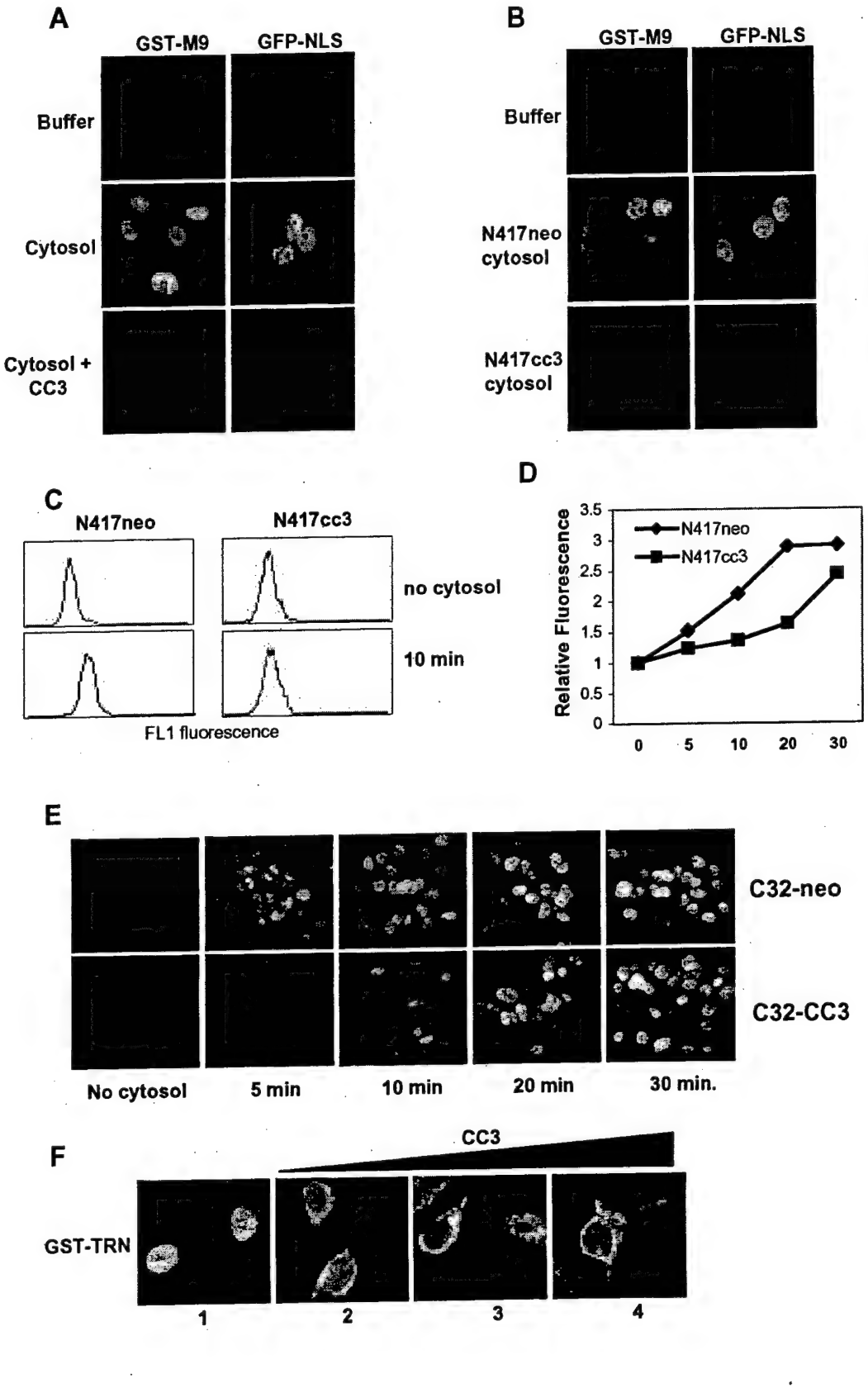
1 2 3 4 5

Figure 3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GST-M9
GFP-NLS
Buffer
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N417cc3 cytosol
C
N417neo
N417cc3
no cytosol
10 min
FL1 fluorescence
D
N417neo
N417cc3
Relative Fluorescence
3.5
3
2.5
2
1.5
1
0.5
0
0
5
10
20
30
E
C32-neo
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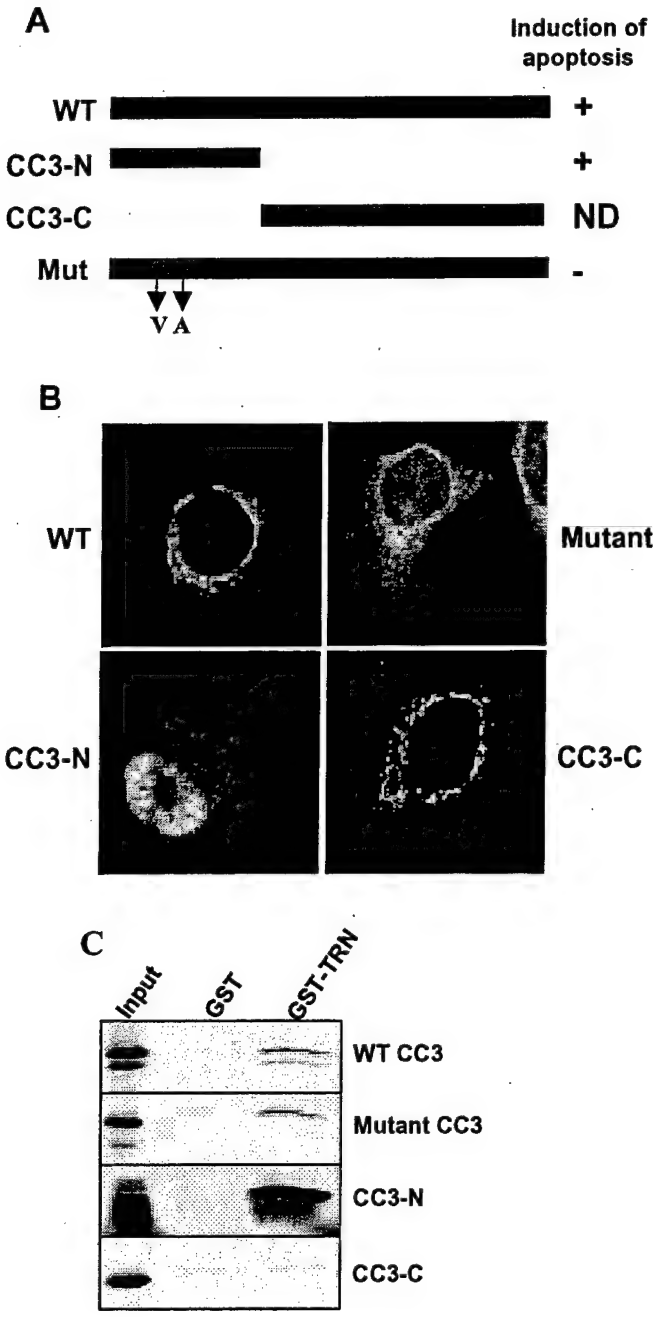
20 min
30 min.
F
CC3
GST-TRN
1
2
3
4

Figure 4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Induction of apoptosis
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CC3-N
+
CC3-C
ND
Mut
-
V A
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Mutant
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CC3-C
C
Input
GST
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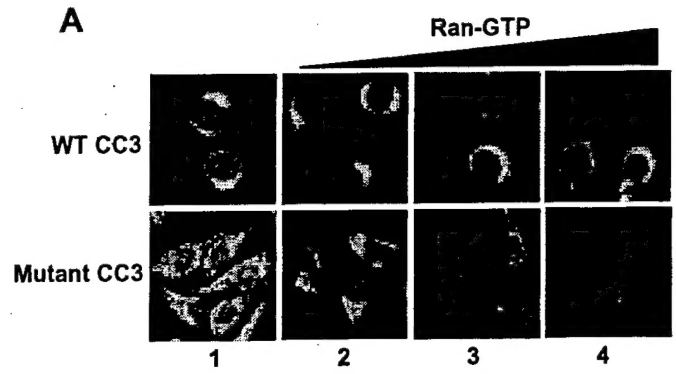
WT CC3
Mutant CC3
CC3-N
CC3-C

Figure 5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WT CC3
Mutant CC3
1
2
3
4

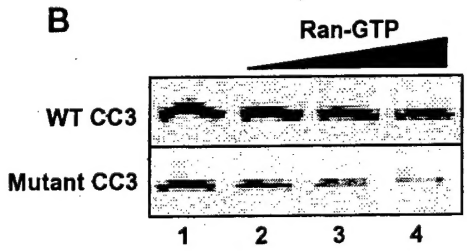
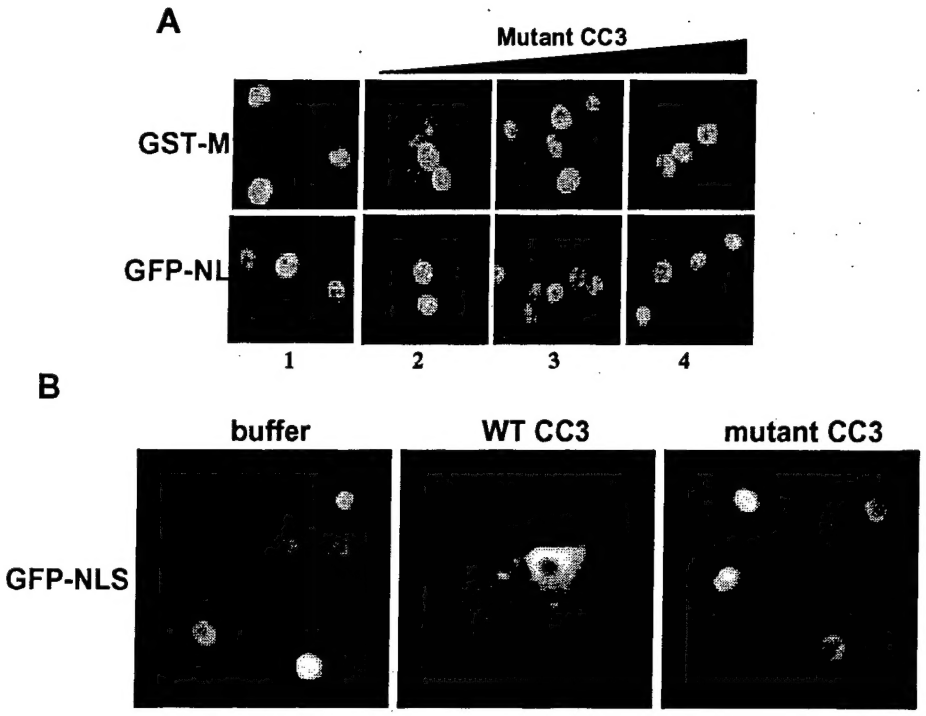

B
Ran-GTP
WT CC3
Mutant CC3
1
2
3
4

Figure 6



C

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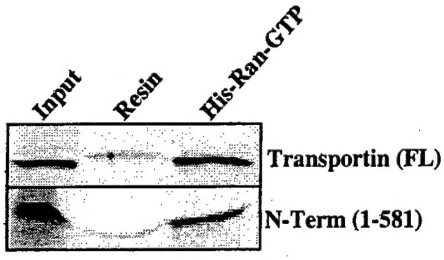
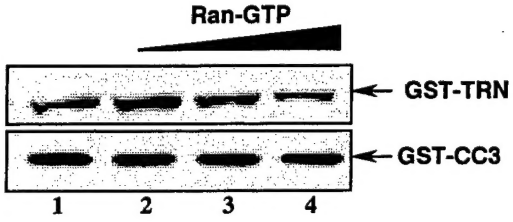
SENP2   1 MYRWLVRIL GTIFRFCDR  SVPPA   RALL  KRRRSDST
CC3   181   EWLVRKLFGSLPDSWARGHSVPVVTVVRAMLNNVVRPRDKQ
```

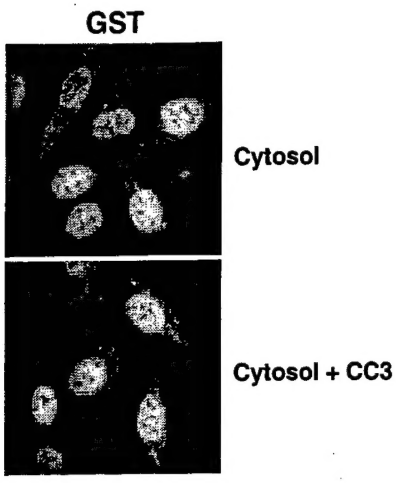
Figure 7



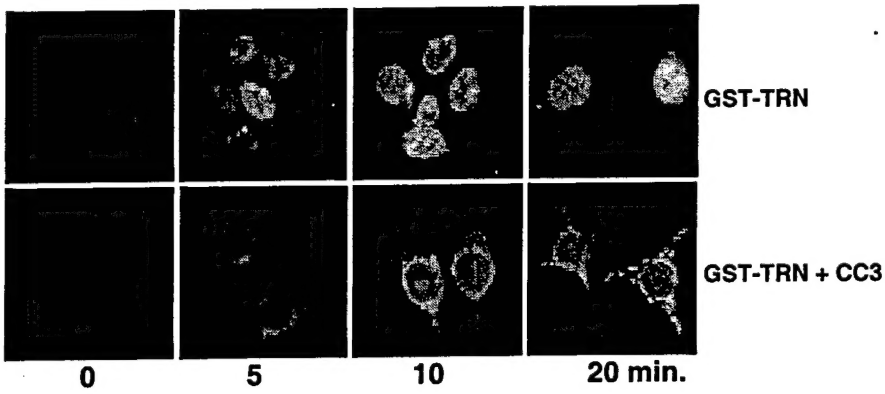
Supplemental Figure 1. *In vitro* translated transportin, either full length or the N-terminal portion, binds RanGTP *in vitro*. *In vitro* translated transportin (FL) or its N-terminal fragment (1-581) were incubated with His-RanQ69L for 1 hour at 4°C. Complexes were recovered with nickel-agarose, resolved by SDS-PAGE and transportin was detected by autoradiography.



Supplemental Figure 2. Interaction of CC3 with nuclei is not inhibited by excess of RanGTP. Nuclei purified from N417 cells were mixed with 1 μM GST-CC3 or GST-transportin alone (lanes 1) or in the presence of 1, 3 and 17 μM RanQ69L (lanes 2-5) for 15 minutes at room temperature. After sedimentation the nuclei-containing pellets were resolved by SDS-PAGE and bound proteins were detected by Western blotting with anti-GST polyclonal antibodies.



Supplemental Figure 3. CC3 does not impede passive diffusion of small proteins through the nuclear pore. Purified GST at 0.5 μM was added to permeabilized Hela cells in transport buffer alone or in the presence of 8 μM CC3. After incubation for 20 minutes at room temperature, cells were fixed and permeabilized with Triton X-100. The cellular localization of transportin was examined by immunofluorescent microscopy using polyclonal anti-GST antibodies and anti-rabbit IgG-FITC.



Supplemental Figure 4. Time course analysis of the inhibition of nuclear translocation of transportin by CC3. GST-transportin at 0.5 μM was added to permeabilized Hela cells in transport buffer with or without CC3 at 8 μM CC3. Cells were fixed at indicated times after addition of transport mixture. The cellular localization of transportin was examined by immunofluorescent microscopy using polyclonal anti-GST antibodies and anti-rabbit IgG-FITC.